An end-joining repair mechanism in *Escherichia coli*

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Bridging broken DNA ends via nonhomologous end-joining (NHEJ) contributes to the evolution and stability of eukaryote genomes. Although some bacteria possess a simplified NHEJ mechanism, the human commensal *Escherichia coli* is thought to rely exclusively on homology-directed mechanisms to repair DNA double-strand breaks (DSBs). We show here that laboratory and pathogenic *E. coli* strains possess a distinct end-joining activity that repairs DSBs and generates genome rearrangements. This mechanism, named alternative end-joining (A-EJ), does not rely on the key NHEJ proteins Ku and Ligase-D which are absent in *E. coli*. Differently from classical NHEJ, A-EJ is characterized by extensive end-resection largely due to RecBCD, by overwhelming usage of microhomology and extremely rare DNA synthesis. We also show that A-EJ is dependent on the essential Ligase-A and independent on Ligase-B. Importantly, mutagenic repair requires a functional Ligase-A. Although generally mutagenic, accurate A-EJ also occurs and is frequent in some pathogenic bacteria. Furthermore, we show the acquisition of an antibiotic-resistance gene via A-EJ, refuting the notion that bacteria gain exogenous sequences only by recombination-dependent mechanisms. This finding demonstrates that *E. coli* can integrate unrelated, nonhomologous exogenous sequences by end-joining and it provides an alternative strategy for horizontal gene transfer in the bacterial genome. Thus, A-EJ contributes to bacterial genome evolution and adaptation to environmental challenges. Interestingly, the key features of A-EJ also appear in A-NHEJ, an alternative end-joining mechanism implicated in chromosomal translocations associated with human malignancies, and we propose that this mutagenic repair might have originated in bacteria.

bacterial adaptability | accurate repair | DNA repair | genome evolution | horizontal gene transfer

DNA double-strand breaks (DSBs) are a potent force in driving genome evolution. In mammalian cells, most DNA rearrangements and insertions result from the prevalent DSB repair mechanism, nonhomologous end-joining (NHEJ) (1, 2). The main evolutionary strength of NHEJ is its ability to join unrelated DNA ends and create new genomic combinations between sequences that do not share homology. The complexity of the NHEJ machinery increases in higher eukaryotes where several proteins are implicated in processing and synapsis of the broken ends, compared to a simpler apparatus in lower eukaryotes. The "core" components Ku70, Ku80, DNA-dependent protein kinase catalytic subunit and the complex Ligase4/XRCC4, and other proteins, including Cernunnos/XLF and Artemis, are committed to ensure efficient NHEJ (2), which is also implicated in the repair of DSB-mediated rearrangements in Ig genes in mammals (3). Recently, alternative end-joining mechanisms (A-NHEJ), Ku-, Ligase4-, XRCC4-, or DNA-PK-independent, have been described in eukaryotes, that are less efficient and/or less accurate than classical NHEJ (4–8). Notably, A-NHEJ, reported in mammals, has been associated with oncogenic translocations in lymphomas (4, 7, 8).

Differently from eukaryotes, bacteria are less proficient in, or lack, NHEJ (9). Some bacteria, including phylogenetically distant *Mycobacteria* and *Bacillus subtilis* possess a rudimentary NHEJ machinery consisting of two proteins, namely the multifunctional ATP-dependent ligase-D and Ku (10, 11) that collectively protect, process, and ligate DNA ends. Bacterial NHEJ, whose mechanism was identified from studies on the repair of linearized plasmids transfected into bacteria (10, 12), can promote survival to DSBs during nondividing phases (dormant and stationary phases, dessiccation) in *Mycobacteria* (13, 9) and sporulation in *B. subtilis* (14, 15). Indeed, under these conditions, homologous recombination (HR), the otherwise exclusive bacterial repair mechanism that relies on the presence of homologous sequences, is expected to be largely ineffective due to the limited number of bacterial chromosomes.

Other bacteria, like the prevalent gut commensal *Escherichia coli* where Ku-like and Ligase-D-like proteins have not been found, are generally accepted to be end-joining-free (9) and to rely only on recombination-mediated mechanisms to repair DNA breaks and incorporate exogenous sequences. Indeed, *E. coli* is used as negative control for NHEJ experiments in other bacteria (16). Here, we investigated whether *E. coli* repairs DSBs via end-joining, independently of conservative homologous recombination, and whether this affects the evolution of its genome.

Results

The occurrence of NHEJ in bacteria was finally demonstrated by the ability of *Mycobacteria* to repair DSB substrates in vivo and by the molecular analysis of the repair events (10, 12). We employed a similar strategy to analyze end-joining in *E. coli*. A variety of DSB-substrates, pSK plasmids linearized to yield noncomplementary ends for end-processing, were delivered to *E. coli* to test end-joining and molecularly analyzed after repair occurred (Fig. 1 *A–C*). The pSK-Cm plasmid, whose intervening chloramphenicol (Cm)-resistance gene is removed after formation of two DSBs, was used to identify accurate repair events at compatible, blunt ends (Fig. 1*B*; modified from ref. 12). We show that laboratory (TG1) and pathogenic (CFT073, 536) *E. coli* strains repair DSBs by bridging DNA ends and with a frequency of 0.35–3.90 $\times$ 10^{-5}, with the highly virulent uropathogenic strain CFT073 showing the highest efficiency (Fig. 1*D*).

Clonal analysis of repair events showed that, surprisingly, the efficiency of end-joining in *E. coli* is independent of the substrate ends (Fig. 1*E*). Moreover, sequencing of 101 junctions revealed that repair is in most cases unfaithful, and this is characterized by extensive bidirectional end-resection (100/101), overwhelming microhomology use (1-9 nucleotides, mostly < 3nt), and absence of DNA synthesis, (Fig. 2 and Fig. S1), in striking contrast with bacterial NHEJ (10, 12), see below. Thus, an elaborated end-joining activity distinct from NHEJ, and that we name alternative end-joining (A-EJ), acts in NHEJ-deficient bacteria. Molecular junctions generated by A-EJ differ from those of bacterial NHEJ (in *Mycobacterium*) (12), which are characterized by large non-templated nucleotide addition (8/21 in NHEJ versus 0/101 A-EJ), frequent unidirectional resection (9/21 in NHEJ versus 0/101 in A-EJ), and no usage of microhomology for deletional repair (0/21 in NHEJ versus 95/101 in A-EJ). We also observed faithful joining of DSBs at compatible ends, with particularly high fidelity in

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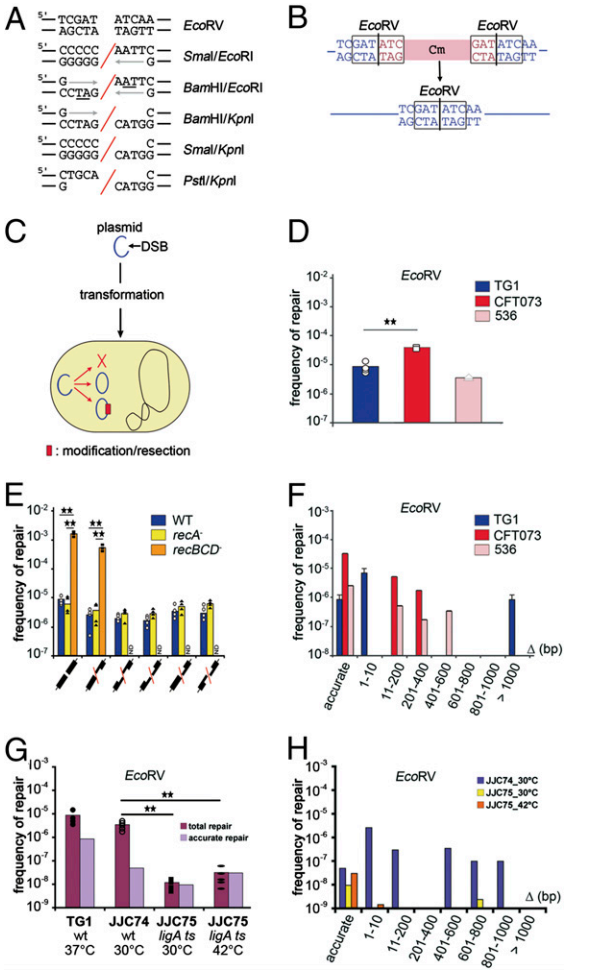


Fig. 1. End-joining in pathogenic and laboratory *E. coli* strains. (*A* and *B*) Plasmid substrates for end-joining. Five substrates with noncompatible ends (crossed by a red bar; a combination of 5′ overhangs, 3′ overhangs, and blunt ends) and one substrate with compatible ends (*Eco*RV) are shown. Microhomologies are underlined. A gray arrow indicates the direction of DNA synthesis. (*C*) Experimental paradigm. The linearized plasmid (open circle), transformed in *E. coli*, is repaired (recircularized) either accurately (only at compatible DNA ends, intact circle) or inaccurately (at each type of DNA ends, circle with box). Cross indicates degradation and loss of the end-joining substrate. (*D*) End-joining in pathogenic (strain 536, CFT073) and laboratory (TG1) *E. coli* strains. Two asterisks, $P \leq 0.01$, according to Student's *t* test. (*E*) The efficiency of repair is independent of the type of DNA ends and of the homologous recombination protein RecA. Increased efficiency of plasmid end-joining in the absence of the RecBCD exonuclease (reduced substrate degradation). (*F*) Distribution of repair events. On the *x* axes are indicated accurate events or the size of deletion (Δ). Error bars (when not visible they are smaller than the drawn line) represent the SD of two to four independent experiments. (*G*) Frequency of repair of the EcoRV substrate (blunt ends) in wild type and *ligA ts* strains at permissive (30 °C) and nonpermissive (42 °C) temperature, compared with the previously analyzed TG1 strain. The *ligA ts* mutant is impaired in end-joining by two orders of magnitude. Essentially accurate repair events occur in the *ligA ts* strain. Two asterisks, $P \leq 0.01$, according to Student's *t* test. (*H*) Distribution of repair events in wild type and *ligA ts* mutant strains.

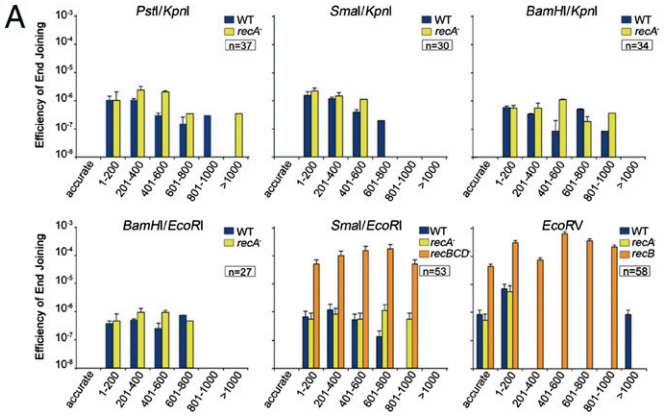
pathogenic strains tested here (82.6% in CFT073 and 71.4% in 536 strain, and 9.8% of accurate events in TG1 and 1.4% in the laboratory strain JJC74, see below). Taking into account the repair efficiency, which varies among these strains, the efficiency of accurate events is at least 3- to 38-fold higher in pathogenic strains tested here (Fig. 1*F*). Sequence analysis (Fig. S2) reveals that in pathogenic strains, accurate events replace the mutagenic repair involving microdeletions (1-10 bp) and deletions >1,000 bp observed in TG1. This finding indicates that some strains carry an improved A-EJ mechanism that promotes precise joining by limiting short- and long-range exonuclease activity. The CFT073 strain has been fully sequenced, but no Ku-like protein or other known NHEJ factor appear to be coded by this strain (17). However, the genome of CFT073 contains insertions islands, compared to the K12-derivative laboratory strain (17), which may code for unidentified proteins that promote faithful end-joining.

To investigate further the mechanism underlying A-EJ, we analyzed end-joining repair in TG1 *recA*$^-$ and *recBCD*$^-$ strains. We report that the efficiency of A-EJ and the types of junctions (sequencing, $n = 86$) are independent of the recombination factor RecA, indicating that no recombination-dependent mechanism is involved (Fig. 1*E* and Fig. 2). However, A-EJ is stimulated approximately 200-fold in the absence of RecBCD complex, which has exonuclease activity. We reasoned that this increase is due to reduced substrate degradation and loss. Sequencing of junctions shows that all repair events are overrepresented in *recBCD*$^-$ strains ($n = 52$), compared with wild-type and *recA*$^-$ strains, in particular events with 200–800 bp deletion (Fig. 2 and Fig. S1), confirming that a larger number of substrates is available when the recBCD complex is absent. Thus, the efficiency of A-EJ appears to depend on the control of exonuclease activities. The type of junctions generated in mutant strains is otherwise remarkably similar to that of the WT strain, namely mutational repair associated with extensive bidirectional end-resection (138/138, cumulated *recA*$^-$ and *recBCD*$^-$), large use of microhomology (129/138) and limited DNA synthesis (1/138).

To gain further insight into the mechanism, we analyzed which DNA ligase activity is involved in A-EJ. In the absence of ATP-dependent LigD and LigC, *E. coli* can rely on the NAD$^+$-dependent ligases LigB and the essential LigA, an enzyme involved in the ligation of the Okazaki fragments, for joining DNA breaks. Repair of linearized plasmids performed in the absence of LigB (*yicF*$^-$ strain) showed that this ligase in not involved in A-EJ (Fig S3*A*). On the contrary, a temperature-sensitive (*ts*) mutation of *ligA* (18) (strain JJC75), results in a 2-log reduction in A-EJ at both permissive and nonpermissive temperature (Fig. 1*G*), compared to the parental strain (JJC74) and TG1, both carrying WT *ligA* (Table S2 and Fig S4). Thus, the *ts* mutation even at permissive temperature impairs the end-joining activity of LigA. Interestingly, residual joining events in this mutant are essentially accurate (Fig. 1 *G* and *H* and Fig. S5), and they appear as frequent as is accurate repair in the WT JJC74 strain. These results indicate than LigA is an important factor influencing the fidelity of A-EJ.

To assess whether A-EJ also repairs chromosomal DSBs, we inserted in the bacterial chromosome two I-*Sce*I endonuclease sites separated by 2.9 kbp, either in the same (compatible ends) or in the opposite (noncompatible ends) orientation, and flanking a suicide cassette (Fig. 3 *A*–*C*). The cassette consists of the suicide gene *ccdB* under the control of an arabinose (Ara)-inducible promoter and of *aadA7* that codes for resistance to spectinomycine (Sp). Expression of the endonuclease I-*Sce*I generates two DSBs and results in the removal of the suicide cassette. Repair of the chromosome allows the growth of Sp-sensitive bacteria on Ara plates. The repair of two distant chromosomal DSBs is much less efficient than the repair of a single DSB also in NHEJ-proficient mammalian cells (frequency of 10^{-2}-10^{-6} and of approximately 10^{-1}, respectively) (19, 20, 21, 22). Nevertheless this observation, involving loss of an intervening sequence and formation of a new junction, clearly demonstrates the occurrence of end-joining.

We show that in *E. coli*, distant DSBs were repaired by end-joining with a frequency of $2.6–3.2 \times 10^{-5}$ (Fig. 3*D*). The efficiency of repair was comparable for complementary and non-complementary chromosomal ends, confirming this finding with plasmids. Molecular analysis and sequencing of repair events in



B

genotype	repair events	PstI/KpnI	SmaI/KpnI	BamHI/KpnI	BamHI/EcoRI	SmaI/EcoRI	EcoRV
WT	total analysed	19	17	19	15	19	12
	accurate	/	/	/	/	/	1*
	non-templated DNA synthesis	0	0	0	0	0	0
	templated DNA synthesis	0	0	0	0	0	0
	deletions	19	17	19	15	19	11
	MH usage	100%	100%	100%	80.0%	89.5%	91.7%
recA⁻	total analysed	18	13	15	12	13	15
	accurate	/	/	/	/	/	1*
	non-templated DNA synthesis	0	0	0	0	0	0
	templated DNA synthesis	0	0	1	0	0	0
	deletions	18	13	15	12	13	14
	MH usage	100%	84.6%	100%	91.7%	92.3%	93.3%
recBCD⁻	total analysed					21	31
	accurate					/	1*
	non-templated DNA synthesis					0	0
	templated DNA synthesis					0	0
	deletions					21	30
	MH usage					100%	96.8%

Fig. 2. A-EJ repairs distinct types of DSBs. (*A*) Histograms representing the distribution of deletions (sum of both DNA ends) associated with end-joining of DSB-substrates (linearized plasmids) in wild type and *recA*⁻ (in two cases, also in *recBCD*⁻) *E.coli*. "*n*" is the number of sequenced events. (*B*) Summary of sequenced A-EJ repair events. For each substrate, the total number of junctions and their classification into four categories are indicated. Accurate repair is expected only for compatible ends (EcoRV). The percentage of repaired junctions that rely on microhomology is reported for each data set. Slash indicates nonapplicable.

survivor bacteria showed extensive resections, up to approximately 40 kbp, accompanied by joining through microhomology (Fig. 4). On one side, the largest resection halts at 43.6 kbp, before the essential *dnaB* gene that codes for a replicative DNA helicase, and on the other side at 2.7 kbp, before *rrlE* that codes for 23s rRNA. rRNAs are highly transcribed genes and loss of one of them can cause decrease in the growth rate (23). The resection limits appear therefore to be directed more by genetic constraints than by directional nuclease(s) activity.

The efficiency of repair of chromosomal DSBs was not affected in *recA*⁻ bacteria (Fig. 3*D*), showing no involvement of recombination-dependent mechanisms in this process, and this was also the case for the repair of plasmids (see above). In the chromosome, most substrate resections are not lethal and the efficiency of repair in *recBCD*⁻ strains was either not affected (complementary ends) or was reduced (noncomplementary ends) possibly due to inefficient degradation of the nonligatable substrate leading to search for other ligatable ends (Fig. 3*D*). Repair junctions in *recA*⁻ and *recBCD*⁻ *E.*

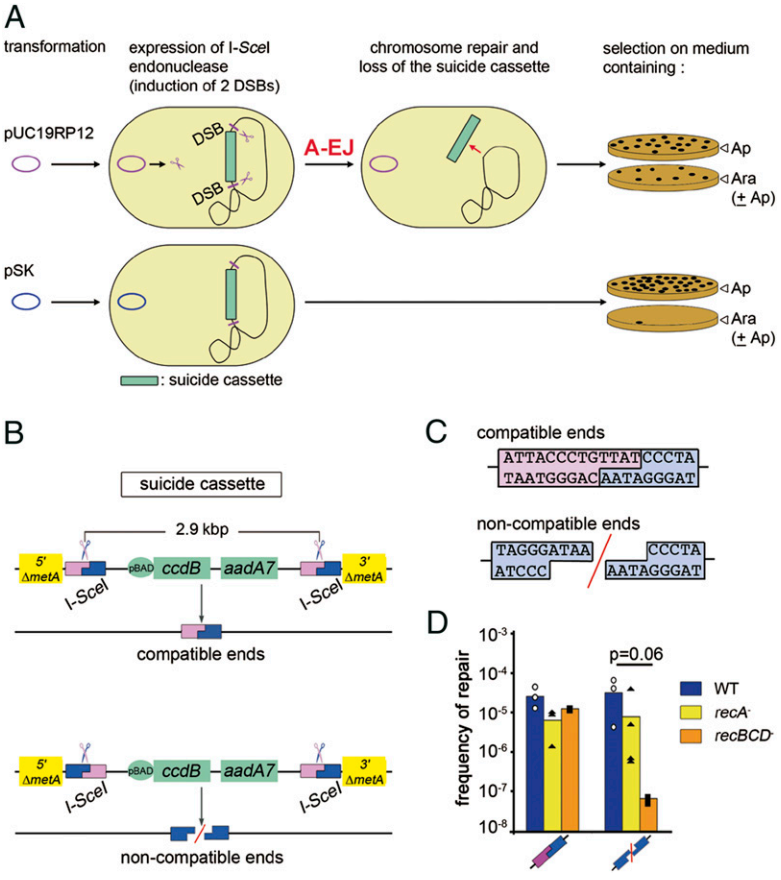


Fig. 3. A-EJ of chromosomal DSBs. (*A*) Experimental paradigm. Plasmid-coded restriction enzymes (scissors) generates two DSBs flanking a suicide cassette (rectangle) in the bacterial chromosome. Bacteria were transformed either with the plasmid pUC19RP12 (which codes for the I-*Sce*I endonuclease) or with the plasmid pSK, as a control. End-joining results in loss of the suicide cassette and growth on selective medium. (*B*) The suicide cassette, inserted in the nonessential *metA* gene, is flanked by two restriction sites for the endonuclease I-*Sce*I (5′ end in pink, 3′end in blue,) either in the same orientation (cassette A, *Upper*) generating compatible ends, or in the opposite orientation (cassette B, *Lower*), generating noncompatible ends. (*C*) Sequence of compatible and noncompatible DNA ends. (*D*) The efficiency of repair is independent of the type of DNA ends and of the homologous recombination protein RecA. *P* value indicated according to the Student's *t* test.

coli appear similar to those in WT bacteria, with microhomologies of 3–8 bp that bridge DNA ends separated by 0.4–12 kbp (Fig. 4*B*). A few accurate events were observed at complementary ends (3/34, 9%, taken together wild type and mutant strains), resulting in a percentage fourfold lower than that observed for the repair of two chromosomal DSBs in NHEJ proficent mammalian cells (9/26, 35%) (21). Inactivation of LigB has no effect in the joining efficiency of chromosomal ends (Fig. S3 *B* and *C*), as it was also the case for A-EJ in plasmid (see above). These findings shows that DNA termini 2.9 kbp apart can be faithfully rejoined by A-EJ. In conclusion, A-EJ is a recombination-independent repair mechanism distinct from NHEJ (Fig. 5*A*).

In eukaryotes NHEJ promotes the insertion of exogenous or heterologous DNA sequences in the genome (virus, transposons, mitochondrial DNA, etc.), thus contributing to genome evolution (1, 24, 25). In bacteria, exogenous sequences are integrated by a variety of homology-dependent mechanisms that select for regions sharing homology with the genome or by site-specific recombination (26). We checked whether A-EJ can insert DNA at DSBs. For this we transformed bacteria with a DNA fragment coding for resistance to the antibiotic kanamycin and not sharing homology with the target sequence. We demonstrate that this DNA fragment is inserted in 1–2% of repair events on plasmids in wild type as well as in *recA*$^-$ and in *recBCD*$^-$ strains (Table S3). Sequencing of seven Km^R strains showed that the insertion took place either through accurate joining or complex end-processing events, revealing not only limited end-resection, but also gap-filling (Fig. 5*B*). Thus, *E. coli* can integrate unrelated, nonhomologous exogenous sequences by end-joining.

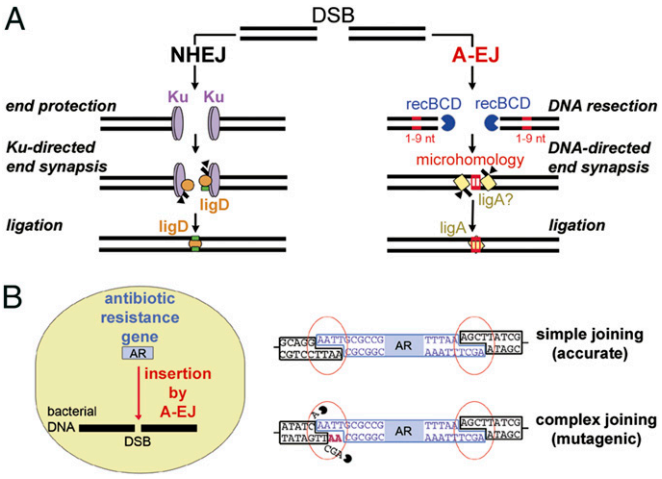


Fig. 5. Insertion of exogenous sequences by A-EJ (*A*) Proposed model for A-EJ (*Right*). Although NHEJ recruits homodimer Ku (ellipse) and LigD (circle) for protection, maturation, and synapsis of the DNA ends, A-EJ relies on the degradation of DNA ends by the RecBCD nuclease/helicase complex (blue incomplete circle). Regions of microhomology (one to nine nucleotides, red strips) are available to promote the synapse between DNA ends. End-ligation likely relies on the essential LigA. (*B*) A-EJ inserts an exogenous, unrelated sequence [AR = antibiotic (Kanamycine) resistance gene] via either accurate joining, or complex end-processing (red letters: newly synthesized bases; diagonal letters: excised bases). Seven events sequenced. In this context, it is conceivable that successful expression of an exogenous gene can also be associated with exonuclease-dependent removal of the original promoter and insertion close to a functional host promoter.

Discussion

The prevalent view is that *E. coli* has no ability to join DNA ends (9), and it has been recently reported that "if it did so, molecular cloning would have had a different story" (27). For this, *E. coli* is used as negative control for NHEJ experiments (16). However, a few events reported in the last 25 years may be attributed to end-joining in *E. coli*, although at that time other mechanisms were proposed. For instance, spontaneous recombination-independent deletions were described in the *E. coli* genome, associated with regions of homology of different sizes (28). The authors found a direct correlation between the size of homology and the frequency of deletion events and they proposed a model based on the slippage between (short) repeated sequences during DNA replication. Moreover, recombination-independent survival of *E. coli* to the activity of a termosensitive endonuclease was reported (29). The repair events were not identified but survival was dependent on the presence of the DNA ligase (29). More recently, Meddows et al. (30) showed chromosomal joining of inducible DSBs via micro (2-5 nt) or longer (16-42 nt) homologies, and also in the absence of homology, mostly at bacterial interspersed mosaic elements (BIME), a class of repeated sequences distributed along the chromosome. Three events that did not occur at BIMEs were attributed to end-annealing or to a different (not identified) mechanism.

Here we show that laboratory and pathogenic *E. coli* strains possess a distinct end-joining mechanism, which we call A-EJ, that repairs DNA breaks and performs elaborate junctions, independently of NHEJ Ku and LigaseD proteins. Differently from *Mycobacterium* NHEJ (12), A-EJ is characterized by frequent bidirectional resection, large usage of microhomology and rare nontemplated DNA synthesis at junctions. These findings, confirmed by the analysis of end-joining of chromosomal DSBs in *E. coli*, show that A-EJ is clearly distinct from NHEJ.

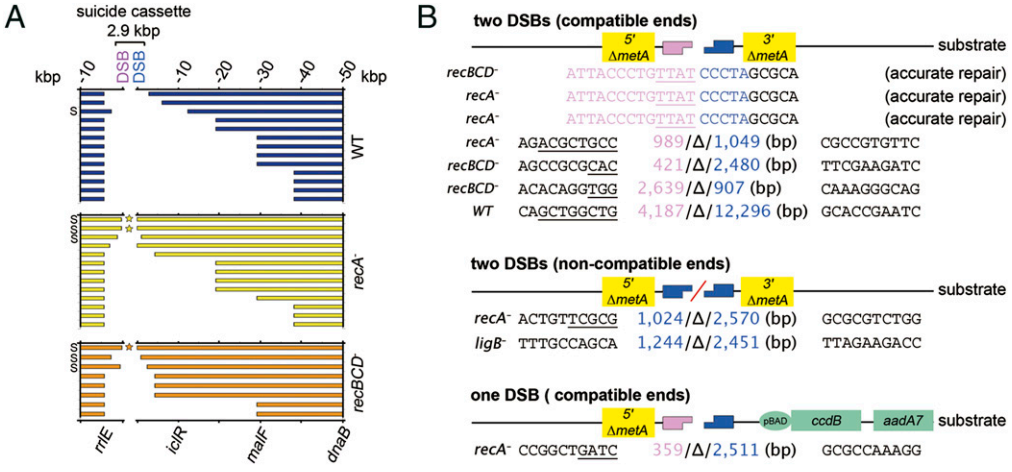


Fig. 4. Repair after A-EJ results in genomic rearrangements. (*A*) Frequent loss of genomic sequences associated with A-EJ in chromosomes. The resection size is reduced in *recBCD*$^-$ *E. coli*. Stars, accurate repair events; "S" sequenced junctions. (*B*) Sequencing of accurate and unfaithful junctions. Joining is characterized by microhomologies of three to eight nucleotides. Unfaithful events are associated with deletions (0.4–12.3 kbp). The lower part indicates a joining event that occurred at a single DSB, and associated with a 2.5-kbp deletion that resected most of the suicide cassette, yielding Ara$^+$ bacteria.

Analysis of repair junctions indicate that in A-EJ unprotected DNA ends by the absence of Ku-like proteins mostly undergo degradation and generate deletions. End-degradation is predominantly, but not exclusively, dependent on the exonuclease activity of the RecBCD complex. The extent of end-resection affects the efficiency of A-EJ, as shown by the higher efficiency of repair of plasmids in *recBCD*$^{-}$ than in WT strain, due to reduced substrate degradation in the mutant. Indeed, in our experimental system, plasmids undergone resection longer than a few hundred bp on each site of the DSB are selected out because of the deletion of key sequences for plasmid replication and selection. This is not the case for chromosomes where much larger deletions are compatible with survival, at least until an essential region is reached, which explains the apparent lack of effect on repair efficiency of the *recBCD* mutation at compatible ends, in spite deletions are generally shorter in the mutant than in the wild type. Importantly, end-resection on long substrates (chromosomes) in turn promotes the search of new ligatable ends by uncovering new microhomologies, and thus favors repair. This appears indeed the case for noncompatible DNA ends that are more efficiently repaired in the resection-prone wild type than in the *recBCD*$^{-}$ strain. We interpret the effect of the RecBCD complex on the repair efficiency of noncompatible chromosome ends as a consequence of the failure to join these ends when unprocessed that in turn promotes RecBCD-dependent end-resection and new homology search. On the contrary, at compatible ends successful joining and/or multiple joining attempts mediated by microhomology may limit the availability of DNA ends to resection. In plasmids, where unlike chromosomes, DNA resection results in large substrate loss (and this is independent of the type of DNA end; see above), the RecBCD complex affects the efficiency of repair both at compatible and noncompatible ends.

A-EJ on plasmids and chromosomes does not depend on LigB, whereas LigA is strongly implicated in the joining process. The *ligA ts* mutant is able to perform Okazaki fragments sealing at permissive temperature (18) although its end-joining activity is dramatically impaired, which allows to functionally uncouple these two processes, at least partially. Our finding is in agreement with the observation that temperature-sensitive *ligA* mutants are abnormally sensitive to UV-induced DNA damage even at permissive temperatures (31). Moreover, the end-joining activity at nonpermissive temperature is in agreement with the initial observation that a strain carrying *lig ts7* (*λhind*$^{-}$) is able to make covalent circles from linear phage λ molecules at 42 °C (18). LigA appears as a key player not only for the efficiency but also for the accuracy of the repair process. In agreement with this notion, Ligase4 has been found to impact on the fidelity of end-joining in mammalian cells, probably by protecting DNA ends from nucleolytic degradation (32).

The large usage of microhomology (93% of cases) in A-EJ supports a model where the synapsis of the two ends strongly depends on the sticking of DNA ends rather than on a protein-driven mechanism, as it is the case for the Ku-dependent NHEJ in *Mycobacterium*, where little or no microhomology is required (12). However, the participation in A-EJ of LigA or other proteins at this stage cannot be excluded. Our finding that a mutant *ligA* is essentially affected in error-prone repair may indicate that LigA interacts with the repair complex at the stage of synapsis formation.

It appears that proteins which participate in A-EJ are also involved in DNA replication and recombination, suggesting that A-EJ evolved with these mechanisms. This is also the case for proteins involved in A-NHEJ in eukaryotes that are implicated in DNA replication and other repair pathways (6, 33). Intriguingly, A-NHEJ is also Ku- and Ligase4 (XRCC4) independent, is associated with end-degradation and is strongly dependent on microhomology (4, 5, 7, 8, 21, 34). Finally, A-NHEJ also uses a NAD+ Ligase. It is therefore tempting to speculate that A-EJ is an ancient repair mechanism that originated in bacteria and that is maintained in higher eukaryotes. Differently from A-NHEJ, however, A-EJ is able to perform accurate repair on compatible ends at a detectable efficiency.

Finally, A-EJ is distinct not only from canonical NHEJ but also from Ku- and LigD-independent NHEJ in *Mycobacterium*, a faithful repair mechanism characterized by end-sealing but not end-processing and that is strictly specific to 3′ overhangs (12). This last mechanism was reported with an efficiency of 10^{-5}, about a 1,000-fold lower that NHEJ in the same organism (extrapolated from ref. 12), but similar to A-EJ in *E. coli* (Table S4). Also, A-NHEJ, analyzed especially at Ig (Ig) loci in mammals, is much less efficient than classical NHEJ (4, 8), indicating an overall low efficiency of alternative end-joining mechanisms. However, Ku-independent A-NHEJ at non-Ig loci is as efficient, although less accurate, as NHEJ (5, 21). A-NHEJ and classical NHEJ coexist, and A-NHEJ is detected when NHEJ is impaired. Interestingly, *E. coli* appears as the only organism that possesses alternative end-joining without possessing the classical NHEJ.

In mammals, A-NHEJ is associated with frequent translocations at Ig loci (7, 8), indicating that repair events due to alternative end-joining, although rare, are not negligible for genome evolution and cell survival. The impact of A-NHEJ on genome rearrangements and stability is underscored by the increase in the frequency of chromosome translocations in the absence of key NHEJ factors (8, 35). Our findings raise the question of the functional role of A-EJ in *E. coli*. In contrast to *Mycobacterium* NHEJ, we found that A-EJ in *E. coli* is not more efficient in early stationary compared to exponential phase (Fig. S6*B*) (13), indicating that A-EJ does not functionally substitute for DSB repair via homologous recombination in nondividing bacteria. In *E. coli*, A-EJ heals DSBs that were not repaired by recombination-dependent mechanisms, thus allowing the survival of bacteria fated to die, and this happens either without or with loss of genetic information. A relevant function of classical NHEJ in eukaryotes is its ability to promote genome evolution by integration of heterologous sequences (1, 24, 25). In bacteria, essentially related and homologous sequences are acquired, and this is due to recombination-dependent mechanisms. Notably, we show that *E. coli* can integrate unrelated, nonhomologous sequences by A-EJ. This process provides an alternative strategy for horizontal gene transfer in bacterial genomes, including the acquisition of antibiotic resistance, and it may be relevant for genome evolution and *E. coli* survival.

Our findings demonstrate that alternative end-joining exists also in the absence of classical NHEJ. We speculate that *E. coli* A-EJ might be the ancestor of alternative end-joining that operates in higher eukaryotes and that may be present also in NHEJ-proficient bacteria. A-EJ in *E. coli* and A-NHEJ in eukaryotes use proteins that are involved in other DNA processing pathways whereas the more efficient NHEJ relies on specific proteins. These observations and the phylogenetic distribution of NHEJ suggest that alternative end-joining precedes NHEJ in evolution. Finally, although less efficient than classical NHEJ, A-NHEJ promotes genome modifications in high eukaryotes that participate in genome evolution and cell survival. Similarly, the ability of A-EJ to integrate exogenous sequences in *E. coli* can contribute to the adaptability of pathogenic and commensal bacteria in hostile and rapidly changing environment.

Materials and Methods

Bacterial Strains. Bacterial strains used in this study are shown in Table S1.

Preparation of End-Joining Plasmid Substrates. The plasmid pBluescript II SK(+) (Stratagene), called here pSK, was digested with a pair of restriction enzymes (Fig. 1*A*), which each recognize a unique restriction site within the multiple cloning site (MCS) to create noncompatible ends. MCS is located in the lacZ α-complementation gene for blue/white color selection of bacterial colonies. The plasmid pSK-Cm, which contains a chloramphenicol resistance gene inserted at the EcoRV site, was digested with EcoRV to remove the Cm

cassette and create blunt ends. The absence or the presence of the Cm gene will discriminate error-free repaired plasmids from background undigested plasmids, respectively (modified from ref. 12). Linearized plasmids, which are end-joining substrates obtained after digestion, were separated from undigested and partially digested molecules by overnight electrophoresis in 1% agarose gel run in Tris–acetate–EDTA buffer, extracted from gel and then purified using a gel extraction kit (Wizard Promega).

Repair of Plasmid DSBs by End-Joining. Laboratory and pathogenic *E. coli* were electroporated with 300 ng of linearized (end-joining substrate) or circular plasmid (control), then plated on selective medium [ampicilline (Ap), X-gal, IPTG]. Bacteria carrying plasmids recircularized by end-joining formed colonies. Blue and white colonies originated from mostly accurate and inaccurate repair, respectively. The end-joining repair efficiency was determined by the ratio of bacterial colonies obtained from linear (repaired) on circular (undigested) plasmids. Values were calculated as the average of three to four independent experiments. To identify the type of repair event, small-scale DNA preparations from bacterial colonies were analyzed by restriction analysis and sequencing. For each of the five end-joining substrates, 12–45 random repair events were sequenced for each bacterial strains (TG1 WT, *recA*$^-$, and *recBCD*$^-$). Twenty-one to 23 repair events of the pSK-Cm plasmid were sequenced from the pathogenic *E. coli* strains CFT073 and 536. Student's *t* test was performed for comparison of wild type vs. *recA*$^-$ and wild type vs. *recBCD*$^-$ bacteria for all DNA substrates.

Repair of Chromosomal DSBs by End-Joining. *E.coli* carrying suicide cassettes in their genome were electroporated with either 300 ng of the plasmid pUC19RP12, that constitutively express the I-*Sce*I nuclease, or with 300 ng of the plasmid pSK (control), both conferring Ap-resistance, then plated either on Ap plates (pSK) or on 0.1% Ara containing plates (pUC19RP12) to induce the expression of the suicide gene *ccdB*. Bacteria that repair the two DSBs by end-joining loose the suicide cassette and become Ara$^+$. Colonies grown on arabinose were then replica plated on Sp containing LB plates to check for the loss of the *aadA7* gene (Sp^S colonies) indicating excision of the complete suicide cassette. The efficiency of chromosomal end-joining repair was determined by the ratio pUC19RP12 Ara$^+$ Sp^S colonies on pSK Ap^R colonies. The efficiency is calculated as the average value of 3–6 independent experiments. Student's *t* test was performed for wild type vs. *recA*$^-$ and for wild-type vs. *recBCD*$^-$ bacteria. To identify the type of repair event, Ap^R Ara$^+$ Sp^S colonies were grown in selective medium and chromosomes were purified using the DNeasy Blood and Tissue kit (Qiagen). In some cases, PCR on colony was performed. Long (until 9–10 kpb) DNA fragments were amplified with the LongAmp Taq PCR kit (Biolabs). The size of deletions associated with end-joining was estimated in some clones by Southern blot analysis, and in all clones by PCR for the presence of short regions increasingly distant from one of the DSB sites. In several cases, the junction was also sequenced.

Insertion of Exogenous DNA by End-Joining. The recipient DNA, plasmid pSK, was digested either by EcoRI and HindIII, or by HindIII, generating noncompatible and compatible ends, respectively, dephosphorylated and purified as described above. The exogenous sequence coded for the resistance to Km had ends compatible with the double digested-recipient DNA. wild type, *recA*$^-$ and *recBCD*$^-$ bacteria were transformed with 300 ng of the recipient (linear pSK) and 300 ng of the exogenous sequence (Km resistance), then plated on Ap Km selective medium. Integration of the exogenous sequence, resulting in Km^R colonies, was confirmed by sequencing. Residual undigested pSW29T plasmids do not confer resistance to kanamycin becasue they are not replicated in TG1 (which lacks the necessary *pir* gene), therefore they do not grow colonies.

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